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(54) Title: DEVELOPMENT OF HIGH-ETHANOL RESISTANT *ESCHERICHIA COLI*

(57) Abstract

The invention relates to a novel selection process to identify novel mutants of *Escherichia coli* KO11, an ethanologenic bacterium, that exhibit the ability to grow and survive in ethanol concentrations beyond that in which the parent *Escherichia coli* KO11 can survive. The new approach, which alternates between selection for ethanol resistance and selection for rapid growth on solid medium containing a high level of chloramphenicol, resulted in strains which are potentially useful for ethanol production.

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DEVELOPMENT OF HIGH-ETHANOL RESISTANT *ESCHERICHIA COLI*

BACKGROUND OF THE INVENTION

The fermentation of waste paper and other lignocellulosic products, such as crop residues, into ethanol offers the opportunity to reduce environmental waste problems and reduce reliance on petroleum-based automotive fuels. Genetically engineered bacteria, such as *Escherichia coli* KO11 (U.S Patent No. 5,000,000) and *Klebsiella oxytoca* P2 (U.S Patent No. 5,424,202), have been developed which convert both pentose and hexose sugars, produced by the hydrolysis of hemicellulose, into ethanol.

Relative to yeast, such as *Saccharomyces*, which is currently used for commercial ethanol production from cane syrup and from hydrolyzed corn starch, *Escherichia coli* KO11 is much less ethanol tolerant. Thus, even though bacteria have been developed that have the ability to convert the sugars from, for example, lignocellulose to ethanol, the problem remains that ethanol tolerance in these bacteria limits both the rate of ethanol production and the final ethanol concentration which can be achieved in the fermentors..

SUMMARY OF THE INVENTION

This invention is based upon the discovery that novel mutants of *Escherichia coli* KO11 exhibit the ability to grow and survive in ethanol concentrations beyond that in which the parent *Escherichia coli* KO11 can survive. The invention is also based upon the discovery of an improved ethanol selection process which

alternates between selection for ethanol resistance in liquid medium and selection for rapid growth on solid medium containing a high level of chloramphenicol. This selection process resulted in novel strains of *Escherichia coli* KO11, as discussed above, which are useful for ethanol production.

It was discovered that, by using this new approach, mutants producing 20% more ethanol and completing fermentation more rapidly than the parental *E. coli* KO11 strain, could be produced. Moreover, the mutants are capable of growth in up to 50 g/L ethanol while the parent is incapable of growth at 35 g/L ethanol. Finally, the mutants show dramatically enhanced survival exposure to 100 g/L ethanol as compared to the parent. These characteristics of the mutants means that the expense of ethanol production from lignocellulosic hydrolysates will decrease by achieving higher ethanol concentrations in shorter times and reducing the costs of nutrients, capital equipment, product recovery and waste disposal.

In one embodiment, the invention comprises a method for the selection of ethanologenic microorganisms comprising contacting the microorganisms sequentially to a liquid medium and a solid medium, wherein said liquid medium is used to select for ethanol tolerance and said solid medium is used to select for ethanologenic microorganisms having the ability to grow and produce ethanol.

In a preferred embodiment, the invention comprises liquid media containing increasing concentrations of ethanol and solid media containing antibiotics and a

fermentable sugar for use in the above selection process.

In another embodiment the invention comprises an ethanologenic microorganism having the ability to grow in ethanol concentrations of greater than 35 g/L. In a preferred embodiment the ethanologenic microorganism is selected from the group consisting of Erwinia, Klebsiella, Xanthomonas, Zymomonas and Escherichia, specifically *K. oxytoca* and *E. coli*. In a more preferred embodiment the *E. coli* bacterium is selected from the group comprising LY01, LY02 and LY03.

In still another embodiment the invention comprises an ethanologenic mutant having the ability to produce at least 10% more ethanol than the parental bacteria, preferably *Escherichia coli* KO11, under equivalent fermentation conditions.

Furthermore, it has been discolvered that the inactivation of cyclic AMP receptor protein and/or active biosynthetic alanine racemase results in a microorganism with improved ethanol tolerance.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

Figure 1A shows the ability of mutants to grow in increasing concentrations of ethanol relative to the parental *Escherichia coli* KO11.

Figure 1B shows the ability of mutants to survive in 10% ethanol (w/w) relative to the parental *Escherichia coli* KO11.

Figure 2A shows the ability of mutants, relative to the parental *Escherichia coli* KO11, to grow in the presence of glucose and no ethanol.

Figure 2B shows the ability of the LYO1 mutants to grow in varying concentrations of ethanol and constant glucose relative to the parental *Escherichia coli* KO11 strain in 3.5% ethanol and constant glucose.

Figure 2C shows the ability of mutants, relative to the parental *Escherichia coli* KO11, to grow in the presence of xylose and no ethanol.

Figure 2D shows the ability of the LYO1 mutants to grow in varying concentrations of ethanol and constant xylose relative to the parental *Escherichia coli* KO11 strain in 3.5% ethanol and constant xylose.

Figure 3A shows the osmotic tolerance of mutants to increasing concentrations of glucose relative to the parental *Escherichia coli* KO11.

Figure 3B shows the osmotic tolerance of mutants to increasing concentrations of xylose relative to the parental *Escherichia coli* KO11.

Figure 4A shows the ability of mutants to convert 14% glucose to ethanol relative to the parental *Escherichia coli* KO11.

Figure 4B shows the ability of mutants to convert 14% xylose to ethanol relative to the parental *Escherichia coli* KO11.

Figure 5 shows a map of pLOI1531 and subclones. The map is of insert DNA from KO11. The vector is not shown.

Figure 6 is a map of pLOI1534 and subclones.

DETAILED DESCRIPTION OF THE INVENTION

As described above, the invention relates to an ethanologenic mutant having improved ethanol tolerance. In one embodiment the mutant can produce at least 10% more ethanol than the parental bacteria, (e.g. *Escherichia coli* KO11) when grown under equivalent conditions. In another embodiment the mutant can grow in ethanol concentrations which exceed those of the parental microorganism.

A microorganism (e.g., an ethanologenic microorganism), or "mutant" of the subject invention can be produced by the process of (1) contacting the parental microorganism (e.g., an ethanologenic microorganism) with a first liquid medium comprising an aqueous solution comprising ethanol, selecting one or more microorganisms that survive; (2) contacting one or more microorganisms obtained from the preceding step with a solid growth medium for a sufficient period of time to permit growth. This process can be repeated, such as two, three, four or more times to further improve ethanol tolerance. With each repeating step, the concentration of ethanol is incrementally increased. Thus, the microorganism(s) obtained from step (2) can be contacted (step (3)) with a second liquid medium comprising an aqueous solution comprising an amount of ethanol greater than present in said first liquid

medium, selecting one or more microorganisms that survive; and (4) contacting one or more microorganisms obtained from the preceding step with a solid growth medium for a sufficient period of time to permit growth. The microorganisms selected in such a manner have improved ethanol tolerance to the parental microorganisms.

The liquid and solid mediums can contain additional components, as necessary or desirable. For example, the solid medium can include nutrients, such as sources of carbon, sulfur and nitrogen suitable for growth of the parental microorganism. Examples of suitable growth medium include Luria broth and Basal Salts Media. Generally, the solid medium will contain a sugar, such as xylose and/or glucose. This is desirable to ensure that the microorganisms selected form this step can produce ethanol from the sugar source in good to excellent yields (such as possessing the same or better ethanol production ability as the parent microorganism). The medium can also include an antibiotic, e.g. chloramphenicol, tetracycline, or ampicillin and a fermentable sugar.

The liquid media include an aqueous solution of ethanol. In addition, the media can, optionally, contain nutrients as well, including a suitable carbon, sulfur and nitrogen source. The liquid medium can also contain a buffer to control the pH of the medium. As in the solid media, the liquid media preferably contain sugar, such as xylose and/or glucose. The first liquid medium, second liquid medium or both generally contain at least about 3.5% (by weight) ethanol. The second and subsequent liquid media contain incrementally greater

concentrations of ethanol. For example, the second liquid medium can contain at least about 4% (by weight) ethanol. A third liquid medium (when present) can contain at least about 4.5% (by weight) ethanol.

The microorganisms which can be subjected to the above process can be prokaryotic or eukaryotic and include bacteria, yeasts and fungi. The process is particularly suited for ethanologenic microorganisms (such as ethanologenic bacterium) which comprise one or more enzymes which convert a sugar (such as a pentose (e.g., xylose) or a hexose (e.g., glucose)) to ethanol. Examples of suitable ethanologenic microorganisms comprise one or more nucleic acid molecules which encode alcohol dehydrogenase and pyruvate decarboxylase.

Ethanologenic bacteria comprising one or more nucleic acid molecules which encode alcohol dehydrogenase and pyruvate decarboxylase (as isolated from, for example, Zymomonas species, such as *Zymomonas mobilis*) are known. Also microorganisms that possess xylulokinase, transaldolase, transketolase and xylose isomerase are known (such as those expressed by enteric bacteria, such as *Escherichia coli*). Many micro-organisms have the ability to convert both xylose and glucose to ethanol. Such organisms possess both sets of enzymes. These organisms have been manufactured by recombinant DNA technology by inserting the nucleic acid molecules which encode one set (or operon) of these enzymes into a host cell which, preferably, expresses the second set (or operon) of these enzymes. Table 1 summarizes several enzymes which possess the ability to convert both pentose and hexose to ethanol in good to high yields.

TABLE 1

Bacteria (Plasmid)	Characteristics	Accession Number (Deposit Date)
K. oxytoca M5A1(pLOI555)	Cm^r, pet[B]	ATCC 68564
K. oxytoca M5A1 S1	Cm^r, Ipet[a]	
K. oxytoca M5A1 S2	Cm^r, Ipet[a]	
K. oxytoca M5A1 S3	Cm^r, Ipet[a]	
K. oxytoca M5A1 P1	Cm^r, Ipet[a]	
K. oxytoca M5A1 P2	Cm^r, Ipet[a]	
K. oxytoca M5A1 B1	Cm^r, Ipet[a]	
E. coli KO11	frd, Cm^r, Ipet[a]	
E. coli (pLOI510)	pet[c]	ATCC 68484 (11/28/90)
E. coli (pLOI308-10)	pet[c]	ATCC 67983 (5/15/89)
E. coli C4 (pLOI292)	pet[c]	ATCC 68237 (2/23/90)
E. coli TC4 (pLOI308-11)	pet[c]	ATCC 68238 (2/23/90)
E. coli TC4 (pLOI297)	pet[c]	ATCC 68239 (2/23/90)
E. coli TC4 (pLOI295)	pet[c]	ATCC 68240 (2/23/90)

a Ipet refers to the integration of *Z. mobilis pdc* and *adhB* genes into the chromosome.

b pet refers to the presence of *Z. mobilis pdc* and *adhB* genes in plasmid pLOI555.

c pet refers to the presence of *Z. mobilis pdc* and *adhB* genes in the indicated plasmid.

Cm^r is the an E. coli shuttle vector carrying the *cat* gene.

A more detailed description of these and other related recombinant organisms, as well as the techniques and materials used in their preparation can be found in, for example, United States Patent Nos. 5,028,539 to Ingram *et al.*, 5,000,000 to Ingram *et al.* 5,424,202 to Ingram *et al.*, 5,487,989 to Fowler *et al.*, 5,482,846 to

Ingram *et al.*, 5,554,520 to Fowler *et al.*, 5,514,583 to Picataggio, *et al.*, copending applications having U.S.S.N 08/363,868 filed on December 27, 1994, U.S.S.N. 08/475,925 filed on June 7, 1995 and U.S.S.N. 08/218,914 filed on March 28, 1994 and standard texts such as, Ausubel *et al.*, *Current Protocols in Molecular Biology*, Wiley-Interscience, New York (1988) (hereinafter "Ausubel *et al.*"), Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second and Third Edition, Cold Spring Harbor Laboratory Press (1989 and 1992) (hereinafter "Sambrook *et al.*") and *Bergey's Manual of Systematic Bacteriology*, William & Wilkins Co., Baltimore (1984) (hereinafter "Bergey's Manual") the teachings of all of which are hereby incorporated by reference in their entirety.

Additional microorganisms having improved ethanologenic activity are described in copending application U.S.S.N. 08/834,901, to Ingram *et al.*, filed April 7, 1997, which is also incorporated herein by reference.

Ethanologenic microorganisms, or host cells which can be employed for the insertion of enzymes which convert one or more sugars to ethanol, can be selected from bacteria, yeasts, fungi, or other cells. Suitable bacteria include Erwinia, Klebsiella, Xanthomonas, Zymomonas (such as *Zymomonas mobilis*) and Escherichia. Preferred species include *K. oxytoca* and *E. coli*. Also envisioned are gram-positive bacteria, such as members of the genera Bacillus, for example, *B. pumilus*, *B. subtilis* and *B. coagulans*, members of the genera Clostridium, for example, *Cl. acetobutylicum*, *Cl. aerotolerans*, *Cl. thermocellum*, *Cl.*

thermohydrosulfuricum and *Cl. thermosaccharolyticum*, members of the genera Cellulomanas like *C. uda* and *Butyrivibrio fibrisolvens*. Acceptable yeasts, for example, are of the species of Cryptococcus like *Cr. albidus*, Monilia, and *Pichia stipitis* and *Pullularia pullulans*.

The above microorganisms can be subjected to the selection process of the claimed invention as they occur in nature or after isolation or genetic manipulation, as in mutation or genetic engineering. For example, soil or fecal samples containing microorganisms can be subjected to the described process. In such an embodiment, the ethanologenic properties of the mutated microorganism can be introduced or further improved by inserting one or more enzymes which convert a sugar to ethanol, as described in the above patents. Alternatively, an isolated ethanologenic microorganism with good to excellent ethanol producing properties (such as one or more of the above recombinant microorganisms) are subjected to the above process.

The temperature and pressure of the process are generally not critical but should be selected to ensure viability and growth of the microorganisms. Suitable temperatures can be between about 20° and about 60°C. Pressure will generally be atmospheric. The pH should also be selected towards the ability of the microorganism to remain viable and grow. For example, the pH may generally be selected to be between about 4.5 and 8.0. The tolerance of a microorganism (or members of the species) can be determined readily and frequently are related to the conditions of the microorganism's native environment. Guidance for selecting optimal

conditions for growth can be obtained for example in *Bergey's Manual of Bacteriology*.

The retention time of each step of the process is also not generally critical. The time in which the microorganisms are subjected to the ethanol-containing liquid medium is generally selected such that some, but not all, of the population has died. For example, the step can last between about 8 hours to two weeks. Frequently, several days can be sufficient. Where the step lasts for several days, it may be desirable to exchange or add fresh liquid medium with the same or greater concentration of ethanol to the microorganisms. Likewise, the time for permitting growth of the microorganism on the solid medium is not generally critical and is dependent upon the microorganism. Slow growing microorganisms will require longer retention times than fast growing microorganisms, as generally known in the art. The time is generally long enough to differentiate colonies which are growing better than other colonies. Frequently, several days are sufficient for a fast growing bacteria.

As set forth above, the mutants produced by the claimed process have greater ethanol tolerance than the parental microorganisms. In mutating ethanologenic microorganisms, the microorganisms can have improved ability to produce ethanol, as well. Once the mutants have been isolated, the genetic basis for the mutation can be identified. For example, the chromosome and/or the RNA transcripts produced by the mutant microorganism can be compared to the parent microorganism. This can be done through hybridization technology, as described generally in Sambrook, *et al.* and Ausubel, *et al.*

Once the genetic basis of the improved mutant has been identified, the mutation can be repeated or an equivalent produced through genetic engineering. For example, where the mutation is based upon the inactivation of a gene or genes, then a recombinantly produced equivalent can be made by deleting the gene, deleting the regulatory sequences of the gene or targeting a site-specific mutation to shift the reading frame or remove an active site of the gene, as described in Ausubel, *et al*. and Sambrook, *et al*. In any event, the result is the inability of the microorganism to express an active gene product. Where the mutation is based upon the increased expression of a gene, then recombinantly produced equivalent can be made by substituting the native promoter with a stronger promoter of the gene, adding an enhancer or introducing more copies of the gene. In yet another embodiment, where the mutation is based upon an activity caused by a mutation in the coding region of a gene, a recombinantly produced equivalent can be prepared by introducing the mutated sequence under the control of a promoter region recognized by the host cell.

In the examples below, it has been discovered that mutants which do not express active cyclic AMP receptor protein, active biosynthetic alanine racemase or both have increased ethanol tolerance. Thus the invention includes microorganisms of increased ethanol tolerance wherein the microorganism does not express active cyclic AMP receptor protein, active biosynthetic alanine racemase or both. This can be achieved, for example, by such molecular biology techniques as site-specific mutagenesis and knocking out the gene, also known as

"knock outs." This can be accomplished, for example, by homologous recombination, described in Ausubel, *et al.* and Sambrook, *et al.*

Microorganisms which do not express active cyclic AMP receptor protein and/or active biosynthetic alanine racemase can be particularly suitable host cells for expressing enzymes (e.g., recombinantly) which convert a sugar to ethanol. Suitable microorganisms for use in the invention are as discussed above. Preferred microorganisms are bacteria, particularly gram-negative bacteria (e.g., enteric bacteria) such as *Escherichia coli*, *Erwinia chrysanthia* and *Klebsiella oxytoca*. Particularly preferred microorganisms include those described in the U.S. Patents and applications to Ingram, *et al.* and Picataggio, *et al.*, above.

The invention further relates to methods of using the ethanol tolerant microorganisms described herein. Microorganisms which have increased ethanol tolerance can be ethanologenic or not ethanologenic. Ethanologenic microorganisms can be used in methods of producing ethanol, employing processes generally known in the art. Examples of suitable ethanol-producing processes include those described in the above patents and application to Ingram, *et al.* and Picataggio, *et al.*, which have been incorporated by reference. An improved process for producing ethanol is described in copending application U.S.S.N. 08/833,435, by Ingram, *et al.*, filed April 7, 1997, which is also incorporated herein by reference. These processes are particularly useful for the production of ethanol from lignocellulosic waste. Other processes for the use of ethanologenic microorganisms include the fermentation of

sugar containing materials to foods and beverages. For example, ethanologenic microorganisms are employed in the manufacture of soy sauce, sake, beer and wine. As such, this invention can be employed to further improve the activities and ethanol tolerance of the microorganisms employed in these processes.

Microorganisms of the invention which are not ethanologenic or slightly ethanologenic are particularly useful as host cells for inserting nucleic acid molecules which encode one or more enzymes which catalyze one or more reactions in the glycolytic pathway, or other pathway which converts sugar to ethanol. That is, such microorganisms are particularly useful in the production of ethanologenic microorganisms through recombinant DNA technology.

The invention will now be illustrated by one or more non-limiting examples.

EXEMPLIFICATION

Methods and Materials

The methods and materials described below were used in carrying out the work described in the examples which follow. For convenience and ease of understanding, the methods and materials section is divided into sub-headings as follows.

Bacterial Strains and Media

E. coli KO11 was used in these studies. This is an ethanol producing derivative of *E. coli* B in which the *Zymomonas mobilis* genes for ethanol production *(pdc, adhB)* have been integrated into the chromosome immediately upstream from chloramphenicol acyl

transferase *(cat)* (U.S. Patent No. 5,424,202). In this strain, resistance to chloramphenicol (600 mg liter^{-1}) was used to select for high level expression of *pdc* and *adhB*. Cultures were grown in modified Luria broth containing per liter: 5 g NaCl, 5 g Yeast Extract, 10 g Tryptone, 40 or 600 mg chloramphenicol, and 20-140 g of fermentable carbohydrate.

Stock cultures of alcohol-resistant mutants were maintained on solid medium containing glucose (20 g liter^{-1}), chloramphenicol (600 mg liter^{-1}), isopropanol (10 g liter^{-1}), and agar (15 g liter^{-1}). Ethanol was added to the broth on a weight basis to prepare a stock solution (100 g kg^{-1}) which was diluted as necessary. Broth containing ethanol was filter sterilized using Nalgene 50 mm, 0.45μm bottle top filters (SFCA).

E. coli DH5a was used as a host for the construction of pUC18-based plasmids. This strain was grown in modified Luria broth without added carbohydrate. Ampicillin (50 mg liter^{-1}) was used for selection as appropriate.

Enrichment and Selection of *E. coli* KO11 Mutants

KO11 cultures were transferred daily by diluting 1:20 to 1:200 into 10 ml of fresh broth containing ethanol and glucose (50 g liter^{-1}) in 18x150 mm culture tubes. Tubes were incubated for 24 h at 35°C without agitation. As cultures increased in density during subsequent transfers, ethanol concentrations were progressively increased to select for resistant mutants. Twice weekly, cultures were diluted and spread on solid medium to enrich for ethanol-resistant mutants which grew rapidly and retained high level expression of the

Z. mobilis genes. Colonies on these plates were scraped into fresh broth and diluted. The dilutions were then used as inocula in ethanol-containing broth. KO11 was initially transferred into 3.5% ethanol. After 5 days of sequential transfer, the ethanol concentration in the broth was increased to 4.0%; after 13 days, the ethanol concentration was increased to 4.5%; after 14 days, the ethanol concentration was increased to 5.0%. Dilutions into higher concentrations of ethanol did not appear to yield mutants with further increases in ethanol resistance which were also capable of rapid growth.

Cultures were diluted and spread on solid medium containing chloramphenicol to allow the isolation of mutants. Large raised colonies were individually tested for ethanol resistance in comparison to the parent, KO11. Colonies were transferred to 3 ml of broth and the resulting suspension diluted 60-fold into 13x100 culture tubes containing 0%, 4.5%, and 5% ethanol. After incubation for 24 h at 35°C, cell growth was measured as $O.D._{550nm}$.

Mutants were maintained on plates containing 1% isopropanol and stored at -75°C in 40% glycerol.

Cell Survival in 10% (w/w) Ethanol

Survival of mutant strains was compared to KO11 after dilution into 10% ethanol. Cell suspensions (approximately 0.05 O.D. at 550 nm) were prepared in Luria broth containing glucose (50 g liter^{-1}) by transferring cells from overnight plates. These were preheated to 35°C and diluted at time zero with an equal volume of preheated broth containing 20% ethanol. Dilution into broth lacking ethanol served as a control.

Serial dilutions were spread on solid medium at time zero (no ethanol only), after 0.5 min, and after 5 min. After overnight incubation at 30°C, colonies from appropriate dilutions were counted to determine relative survival as colony forming units (CFU).

Fermentation Experiments

Inocula were grown for 16 h (Beall, D.S. *et al.*, "Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli.*" *Biotechnol. Bioeng. 38*:296-303 (1991)) (30°C) without agitation in Luria broth containing glucose or xylose (50 g/L). Cells were harvested by centrifugation (6000 x g, 5 min, ambient temperature) and added to initiate fermentation to provide 1.0 OD at 550 nm (approximately 330 mg liter^{-1}, dry cell weight). Batch fermentations were carried out at 35°C (100 rpm) in modified 500-ml Fleaker™ beakers containing 350 ml of Luria broth supplemented with glucose or xylose (140 g liter^{-1}). Sugar solutions were sterilized by autoclaving separately. Automatic addition of 2 N KOH was used to prevent acidification above pH 6. Samples were removed to measure cell mass and ethanol. Base consumption and pH were also recorded.

Genetic Methods and Construction of a Genomic Library

Chromosomal DNA was isolated from KO11 essentially as described by Cutting, S.M. and Vander Horn, P.B., Genetic analysis, p. 37-74. *In* C.R. Harwood and S.M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & sons Ltd., Chichester, England (1990). A genomic library of the parental strain, KO11,

was constructed by ligating *Sau*3AI partial digestion products (4-9 kbp fragments) into the *Bam*HI site of pUC18 followed by transformation into DH5α. The resulting library consisted of approximately 8,000 clones. After pooling these colonies, plasmids were isolated to produce a library. Standard procedures were used for the construction, isolation, transformation, and analysis of plasmids (Sambrook et al., 1989).

Isolation of Plasmids Containing Native DNA Fragments from KO11 Which Decrease Ethanol Tolerance by Reverse Complementation

D-cycloserine was used to selectively kill cells capable of growing in 3.5% ethanol (w/w) while allowing non-growing cells to survive. At this concentration of ethanol, mutants continue to grow while the parent remains viable without increasing in cell number. LY02 and LY03 were transformed with the KO11 plasmid library and allowed to grow overnight into colonies. Recombinant colonies of each mutant were harvested by scraping into Luria broth containing glucose (50 g liter^{-1}) and 3.5% ethanol, inoculated to provide 0.1 O.D. at 550 nm, and incubated at 35°C. After 1.5 hr, D-cycloserine (100 mg liter^{-1}) was added and the incubation continued for 4 hours. After 4 h, CFU/ml had dropped by over 95%. Clones harboring putative genes for decreased ethanol tolerance were isolated from these plates.

DNA Sequencing and Sequence Analysis

The QIAprep spin plasmid kit (Qiagen, Chatsworth, CA) was used for plasmid purification. Dideoxy

sequencing was performed using fluorescent primers [forward, 5'CACGACGTTGTAAAACGAC-3' (SEQ ID NO:1); reverse, 5'-ATAACAATTTCACACAGGA-3'(SEQ ID NO:2)] (LI-COR, Lincoln, NE). Extension reactions were performed with a Perkin Elmer GeneAmp PCR System 9600 (Norwalk, CT) using an Excel Sequencing Kit-LC (Epicentre Technologies, Madison, WI) (30 cycles; denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C, and extension for 1 min at 70°C). Extension products were separated and read with a LI-COR DNA Sequencer model 4000L.

Sequences were analyzed using the Wisconsin Genetics Computer Group (GCG) software package and the National Center for Biotechnology Information BLAST network service.

Analytical Procedures

Cell density was measured using a Bausch & Lomb Spectronic 70 spectrophotometer and converted to dry cell weight based on a standard curve for this organism. Ethanol was measured by gas chromatography with n-propanol as an internal standard (Beall et al., 1991) using a Varian Star 3400 CX gas chromatograph.

EXAMPLE 1 - Isolation of Ethanol-tolerant Mutants of *E. Coli* KO11

Using the materials and procedures outlined above, a total of 135 colonies were initially tested for growth in 4.5% (w/w) ethanol, a concentration at which the parental KO11 failed to grow. Forty three colonies were

turbid after 24 h incubation at 35°C and were saved for further testing.

Stability of the ethanol resistance trait was examined by retesting clones which had been stored frozen at -75°C and clones which had been maintained on solid medium lacking ethanol.

EXAMPLE 2 - Testing Mutants of *E. Coli* KO11 for Ethanol Tolerance

As shown in Figure 1A, after 24 h, the mutants were much more resistant to ethanol than the parent KO11. Figure 2B, Figure 2D, and Table 1A and 1B compare initial growth upon dilution into media containing various concentrations of ethanol. In the absence of ethanol, KO11 appeared to grow slightly better than the mutants both with glucose and with xylose as the fermentable sugar as shown in Figures 2A and 2C. However, KO11 was unable to grow in 3.5%(w/w) ethanol while all mutants grew in ethanol concentrations of up to 5% (w/w). Ethanol tolerance was also compared using other fermentable sugars which may be of interest for fuel ethanol production: lactose, arabinose, and mannose, galactose, sucrose and raffinose. KO11 growth was consistently above that of the mutants in the absence of ethanol. However, KO11 failed to grow in ethanol concentrations above 3%(w/w) while the mutants grew in 5% ethanol with all sugars tested.

Next, cell survival during exposure to 10% ethanol was examined (Figure 1B). All mutants tested were more resistant than KO11. This difference was particularly dramatic for 0.5 min exposure where 63-84% of the

mutants retained colony forming ability while less than 10% of KO11 survived.

The stability of the ethanol-tolerance trait was also examined. LYO1, LYO2, LYO3, and LYO4 were transferred daily on solid medium lacking alcohol for 30 days. Cells were then used to inoculate broth cultures containing ethanol as shown in Figures 2C and 2D. The resulting growth curves were identical to those initially determined.

The retention of osmotic tolerance to sugars is essential for the utility of ethanol-tolerant mutants of KO11. As illustrated in Figures 3A and 3B, tolerance to xylose and glucose are similar on a molar basis. With both sugars, KO11 appeared more resistant to osmotic stress than the ethanol-resistant mutants, although this difference was not dramatic. No differences were observed in growth at 48°C, the maximum temperature for growth. Both KO11 and the mutants grew slowly at this temperature.

TABLE 1A Fermentation of 14% glucose to ethanol

				Ethanol Produced[b]		
Strain	Reps n	Cell Mass[a] (g/liter)	Base Consumed (mmoles/liter)	Time (hour)	g/liter	Yield (% theoretical)[c]
LY01	3	4.0 ± 0.78	124 ± 2.7 143 ± 31.6 171 ± 29.2	48 72 96	56.4 ± 2.69 60.3 ± 3.34 61.0 ± 3.45	79.0 ± 3.77 84.5 ± 4.68 85.4 ± 4.83
LY02	7	3.1 ± 0.68	118 ± 21.8 129 ± 57.7 135 ± 63.7	48 72 96	55.4 ± 3.42 60.3 ± 2.86 61.1 ± 1.85	77.6 ± 4.78 84.5 ± 4.01 85.6 ± 2.59
LY03	7	3.6 ± 0.73	124 ± 19.2 148 ± 65.6 153 ± 65.3	48 72 96	53.2 ± 3.39 58.5 ± 3.10 59.6 ± 1.88	74.5 ± 4.75 81.9 ± 4.34 83.4 ± 2.63
LY04	3	3.3 ± 0.77	139 ± 26.5 149 ± 29.4 152 ± 38.3	48 72 96	56.2 ± 1.42 61.5 ± 1.39 61.9 ± 1.69	78.7 ± 1.99 86.1 ± 1.95 86.7 ± 2.37
LY05	1	2.64	171 171 171	48 72 96	54.5 60.0 60.3	76.3 84.0 84.5
LY06	1	3.2	177 229 229	48 72 96	53.6 57.4 57.9	75.1 80.4 81.1
LY07	1	3.2	149 183 183	48 72 96	52.7 58.1 58.6	73.8 81.4 82.1
LY08	1	3.5	166 177 177	48 72 96	54.5 57.3 56.6	76.3 80.3 79.3
LY09	1	3.5	171 211 234	48 72 96	46.1 50.9 52.7	64.6 71.3 73.8

TABLE 1A Fermentation of 14% glucose to ethanol (Continued)

				Ethanol Produced[b]		
Strain	Reps n	Cell Mass[a] (g/liter)	Base Consumed (mmoles/liter)	Time (hour)	g/liter	Yield (% theoretical)[c]
LY10	1	3.5	160	48	54.3	76.1
			177	72	57.6	80.7
			177	96	57.0	79.8
LY11	1	3.0	137	48	51.3	71.8
			194	72	55.6	77.9
			200	96	57.4	80.4
LY12	1	3.7	114	48	56.5	79.1
			131	72	62.1	87.0
			131	96	62.7	87.7
LY13	1	2.5	114	48	57.9	81.1
			234	72	61.9	86.7
			240	96	61.2	85.7
LY14	1	2.8	143	48	53.7	75.2
			200	72	58.6	82.1
			257	96	62.0	86.8
LY15	1	3.8	189	48	51.9	72.7
			217	72	58.0	81.2
			246	96	58.7	82.2
LY16	1	3.2	246	48	40.4	56.6
			309	72	44.6	62.5
			337	96	48.0	67.2
LY17	1	4.1	183	48	57.2	80.1
			189	72	59.4	83.2
			189	96	58.3	81.7
LY18	1	3.7	143	48	52.9	74.1
			171	72	58.6	82.1
			171	96	57.7	80.8

TABLE 1A Fermentation of 14% glucose to ethanol (Continued)

				Ethanol Produced[b]		
Strain	Reps n	Cell Mass[a] (g/liter)	Base Consumed (mmoles/liter)	Time (hour)	g/liter	Yield (% theoretical)[c]
LY19	1	3.1	137	48	52.8	73.9
			189	72	58.1	81.4
			189	96	58.1	81.4
LY20	1	3.3	194	48	40.8	57.1
			251	72	45.3	63.4
			297	96	46.5	65.1
KO11	9	2.8 ± 0.86	151 ± 31.5	48	47.0 ± 1.78	65.8 ± 2.49
			169 ± 29.3	72	50.5 ± 2.69	70.7 ± 3.77
			186 ± 31.7	96	52.7 ± 3.61	73.8 ± 5.05

[a] Cell dry weight, ± s.d.
[b] Corrected for dilution by base
[c] The theoretical yield is 0.51 g ethanol/g glucose.

TABLE 1B Fermentation of 14% xylose to ethanol

Strain	Reps n	Cell Mass[a] (g/liter)	Base Consumed (mmoles/liter)	Ethanol Produced[b] Time (hour)	Ethanol Produced[b] g/liter	Yield (% theoretical)[c]
LY01	4	3.6 ± 0.36	74 ± 4.0	48	51.0 ± 2.09	71.4 ± 2.93
			79 ± 4.7	72	60.9 ± 1.00	85.3 ± 1.40
			79 ± 4.7	96	63.0 ± 1.32	88.2 ± 1.85
			80 ± 4.0	120	63.0 ± 1.20	88.2 ± 1.68
LY02	6	3.3 ± 0.43	75 ± 14.5	48	42.6 ± 5.81	59.7 ± 8.14
			80 ± 14.4	72	56.7 ± 4.90	79.4 ± 6.86
			86 ± 12.2	96	62.2 ± 2.93	87.1 ± 4.10
			89 ± 11.1	120	63.8 ± 1.62	89.4 ± 2.26
LY03	6	3.5 ± 0.34	76 ± 7.8	48	42.1 ± 4.43	59.0 ± 6.20
			87 ± 9.0	72	55.5 ± 4.04	77.7 ± 5.66
			91 ± 10.4	96	60.5 ± 4.16	84.7 ± 5.83
			95 ± 10.3	120	62.9 ± 1.32	88.1 ± 1.85
LY04	2	3.6	88.6	48	44.3	62.0
			91.4	72	56.2	78.7
			97.1	96	60.3	84.5
			97.1	120	62.0	86.8
K011	8	3.1 ± 0.46	81 ± 13.2	48	43.1 ± 2.51	60.4 ± 3.52
			97 ± 14.3	72	52.0 ± 2.08	72.8 ± 2.91
			102 ± 12.8	96	56.8 ± 2.15	79.6 ± 3.01
			105 ± 12.2	120	59.5 ± 2.25	83.3 ± 3.15

[a] Cell dry weight, ± s.d.
[b] Corrected for dilution by base
[c] The theoretical yield is 0.51 g ethanol/g xylose.

EXAMPLE 3 - Fermentation of Sugars to Ethanol

Twenty of the most promising mutants were screened for their ability to produce ethanol from 14% glucose (Figure 4A and Table 2) and 14% xylose (Figure 4B and Table 2) in pH-controlled fermenters. All were superior to the parent strain KO11 in their ability to produce ethanol more rapidly and in their ability to achieve higher final ethanol concentrations. All mutants were also superior to the parent strain KO11 in their ability to achieve higher yields. Mutants LYO1, LYO2, LYO3, and LYO4 were examined in more detail to establish the variability in testing. With all mutants, the cell mass produced during the fermentation of glucose or xylose was consistently higher than that produced by KO11. Base consumed for the neutralization of small amounts of organic acids and dissolved CO_2 was higher for glucose than for xylose due to the higher rate for fermentation. Roughly equivalent amounts of KOH were required to maintain pH 6.0 for both KO11 and the mutants. Addition of base resulted in a small dilution of product. Ethanol values in Table 2 are corrected for this dilution (ethanol produced = measured ethanol *(1000+ 1/2 mM KOH/1000)) to allow the estimation of ethanol yield. After 96 h, the ethanol yield for KO11 with 14% sugar was 74% and 80% of the theoretical maximum (0.51 g ethanol per gram of pentose or hexose) for glucose and xylose, respectively. Strain LYO1 was among the best of the mutants tested. This strain achieved 85% of the maximum theoretical yield from glucose and xylose after 72 h and reached a final ethanol concentration of almost 60 g $liter^{-1}$ (7.5% ethanol by volume).

TABLE 2

	SPECIFIC GROWTH RATE (μ)			
	Organism			
	KO11	LY01	LY02	LY03
Glucose				
0% EtOH	2.50	1.96	2.01	1.83
3.5% EtOH	0.00	0.20	0.19	0.19
4.0% EtOH	nd[a]	0.13	0.12	0.12
4.5% EtOH	nd	0.08	0.08	0.08
5.0% EtOH	nd	0.06	0.06	0.06
Xylose				
0% EtOH	1.40	0.99	1.00	0.98
3.5% EtOH	0.00	0.16	0.15	0.15
4.0% EtOH	nd	0.10	0.09	0.09
4.5% EtOH	nd	0.06	0.05	0.05
5.0% EtOH	nd	0.04	0.05	0.04

EXAMPLE 4 - Isolation of Plasmids Containing Native Genes from KO11 Which Decrease Ethanol Tolerance

After D-cycloserine enrichment for survivors which fail to grow at 3.5% ethanol, a total of 32 recombinants of LYO2 and 40 recombinants of LYO3 were screened in triplicate for growth in Luria broth containing 5% glucose and 4% ethanol. All grew to a lower density after 24 h than the original mutant. Six replicates of the most promising 19 clones were compared simultaneously. Four clones which uniformly resulted in a decrease in ethanol tolerance as compared to LYO2(pUC18) were selected for further study.

EXAMPLE 5 - Identification of Genes Which Reduce Ethanol Tolerance

Sequence analysis of the ends of the inserts in the four selected plasmids revealed that they consisted of two pairs of siblings. The entire *E. coli* genome is now available in the GenBank database. The cloned fragments were readily identified by using the terminal sequences and BLAST network server.

Plasmid pLOI1531 contains a 3.6 kbp fragment of KO11 DNA within the 67.4 min-76.0 min region of the *E. coli* chromosome (GenBank Accession Number U18997). Subclones were prepared and sequenced to confirm gene arrangement in KO11 and to identify the genes which are responsible for a decrease in ethanol tolerance (Figure 5). Only 2 open reading frames were present: *crp* and a large open reading frame of unidentified function (ORF 0696). A comparison of the effects of pUC18, pLOI1531 and deleted clones (pL0I1532 and pL0I1533) on LYO3 indicated that the *crp* gene (cyclic AMP receptor protein) was responsible for the decrease in ethanol tolerance.

pL0I1534 contains a 7.39 kbp fragment of KO11 DNA within the 89.2 min -92.8 min segment (Accession Number U00006 of the *E. coli* chromosome (Figure 6). Deleted derivatives of this clone were also sequenced to confirm gene arrangement and revealed the presence of 3 complete and 2 partial open reading frames: *dnaB'*, *alr*, *tyrB*, *napA(hobH)*, and *uvrA'*. A comparison of deleted derivatives identified the *alr* gene (biosynthetic alanine racemase) as being responsible for decreasing the ethanol tolerance of LYO2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Florida Research Foundation Incorporated

(ii) TITLE OF INVENTION: Development of High-Ethanol Resistant Escherichia Coli

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Lexington
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02173-4799

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/834,900
(B) FILING DATE: 07-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Elmore, Carolyn S

(B) REGISTRATION NUMBER: 37,567

(C) REFERENCE/DOCKET NUMBER: UF97-02.PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 781-861-6240

(B) TELEFAX: 781-861-9540

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACGACGTTG TAAAACGAC 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATAACAATTT CACACAGGA 19

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. An ethanologenic microorganism comprising one or more nucleic acid molecules which encode alcohol dehydrogenase and pyruvate decarboxylase; wherein said bacterium does not express active cyclic AMP receptor protein, active biosynthetic alanine racemase or both.

2. The ethanologenic microorganism according to Claim 1, wherein said microorganism is a bacterium selected from the group consisting of Erwinia, Klebsiella, Xanthomonas and Escherichia.

3. The ethanologenic bacterium according to Claim 2, wherein said bacterium is *Klebsiella oxytoca*.

4. The ethanologenic bacteria according to Claim 2, wherein said bacterium is *Escherichia coli*.

5. The ethanologenic bacteria according to Claim 2 wherein said bacterium comprises a heterologous nucleic acid molecule which encodes *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase.

6. The ethanologenic bacterium according to Claim 5 wherein said *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase are encoded by a nucleic acid molecule isolated from *Zymomonas mobilis*.

7. An ethanologenic *Escherichia coli* which comprises a heterologous nucleic acid molecule isolated from *Zymomonas mobilis* which encodes alcohol dehydrogenase and pyruvate decarboxylase, wherein said bacterium does not express active cyclic AMP receptor protein, active biosynthetic alanine racemase or both.

8. An ethanologenic microorganism produced by the process comprising the steps of:
   (a) contacting an ethanologenic microorganism with a first liquid medium comprising an aqueous solution comprising ethanol, selecting one or more microorganisms that survive;
   (b) contacting one or more microorganisms obtained from step (a) with a solid growth medium for a sufficient period of time to permit growth;
   (c) contacting one or more microorganisms obtained from step (b) with a second liquid medium comprising an aqueous solution comprising an amount of ethanol greater than present in said first liquid medium, selecting one or more microorganisms that survive; and
   (d) contacting one or more microorganisms obtained from step (c) with a solid growth medium for a sufficient period of time to permit growth.

9. The ethanologenic microorganism according to Claim 8 wherein said microorganism is a bacterium.

10. The ethanologenic microorganism according to Claim 9, wherein said microorganism is selected from the group consisting of Erwinia, Klebsiella, Xanthomonas and Escherichia.

11. The ethanologenic microorganism according to Claim 10, wherein said microorganism is *Klebsiella oxytoca*.

12. The ethanologenic microorganism according to Claim 10, wherein said microorganism is *Escherichia coli*.

13. The ethanologenic microorganism according to Claim 10, wherein said microorganism comprises a heterologous nucleic acid molecule which encodes *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase.

14. The ethanologenic microorganism according to Claim 13, wherein said *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase are encoded by a nucleic acid molecule isolated from *Zymomonas mobilis*.

15. A method for producing an ethanologenic microorganism mutant comprising:
    (a) contacting an ethanologenic microorganism with a first liquid medium comprising an aqueous solution comprising ethanol, selecting one or more microorganisms that survive;
    (b) contacting one or more microorganisms obtained from step (a) with a solid growth medium for a sufficient period of time to permit growth;

(c) contacting one or more microorganisms obtained from step (b) with a second liquid medium comprising an aqueous solution comprising an amount of ethanol greater than present in said first liquid medium, selecting one or more microorganisms that survive; and

(d) contacting one or more microorganisms obtained from step (c) with a solid growth medium for a sufficient period of time to permit growth.

16. The method according to Claim 15 wherein said microorganism is a bacterium.

17. The method according to Claim 16, wherein said microorganism is selected from the group consisting of Erwinia, Klebsiella, Xanthomonas and Escherichia.

18. The method according to Claim 17, wherein said microorganism is *Klebsiella oxytoca*.

19. The method according to Claim 17, wherein said microorganism is *Escherichia coli*.

20. The method according to Claim 17, wherein said microorganism comprises a heterologous nucleic acid molecule which encodes *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase.

21. The method according to Claim 20 wherein said *Zymomonas* alcohol dehydrogenase and pyruvate decarboxylase are encoded by a nucleic acid molecule isolated from *Zymomonas mobilis*.

22. The method according to Claim 15, wherein said solid medium contains an antibiotic and a fermentable sugar.

23. The method according to Claim 22, wherein said antibiotic is selected from the group consisting of chloramphenicol, tetracycline and ampicillin.

24. The method according to Claim 15, wherein said first liquid medium, said second liquid medium or both further comprise a sugar.

25. The method according to Claim 24, wherein said first liquid medium, said second liquid medium or both comprise at least about 3.5% (by weight) ethanol.

26. The method according to Claim 25, wherein said second liquid medium comprises at least about 4% (by weight) ethanol.

27. The method according to Claim 26, further comprising steps

(e) contacting one or more microorganism obtained from step (d) with a third liquid medium comprising an aqueous solution comprising an amount of ethanol greater than present in said second liquid medium, selecting one or more microorganisms that survive; and

(f) contacting one or more microorganisms obtained from step (e) with a solid growth medium for a sufficient period of time to permit growth.

28. The method according to Claim 15, wherein said ethanologenic microorganism mutant can grow in ethanol concentrations of greater than 35 g L^{-1}.

29. The method according to Claim 15, wherein said ethanologenic microorganism mutant can produce at least 10% more ethanol than *Escherichia coli* KO11 under equivalent fermentation conditions.

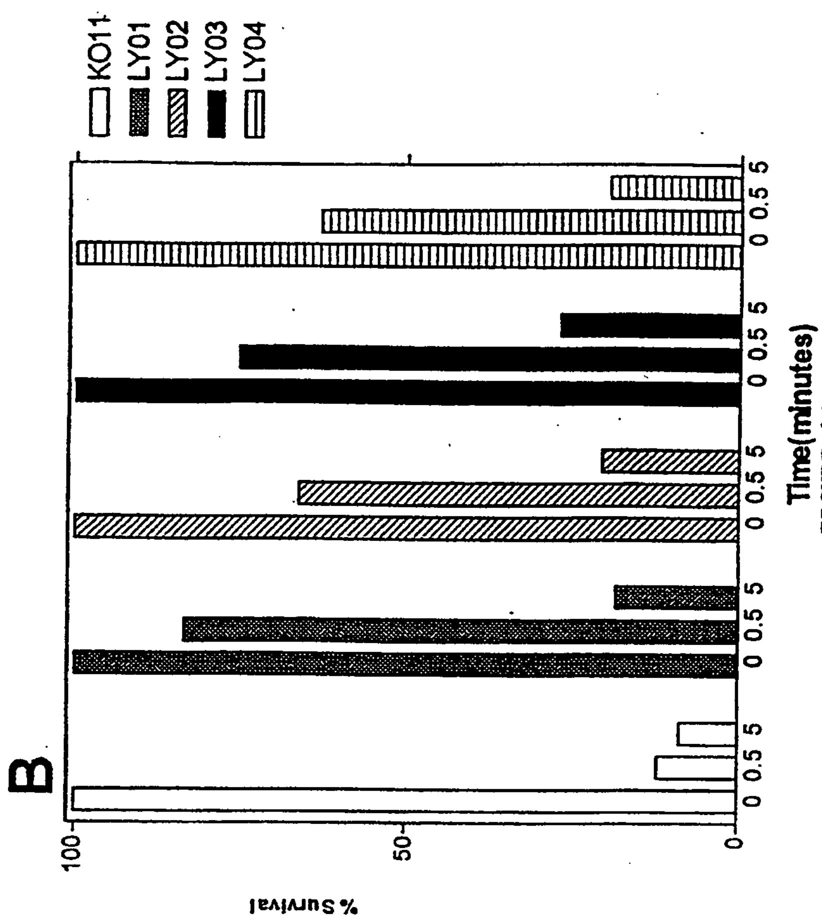


FIGURE 1B

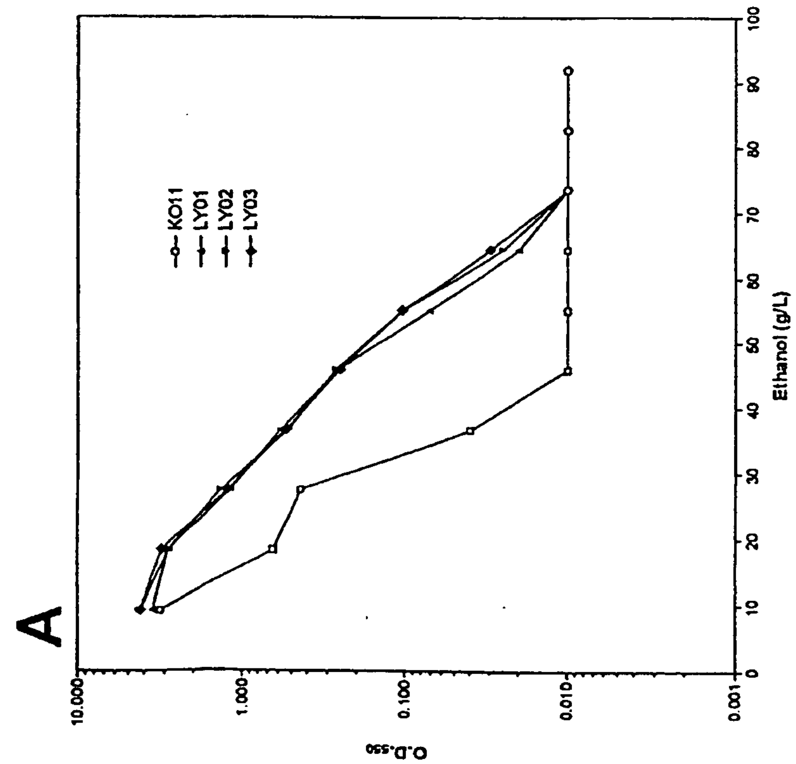


FIGURE 1A

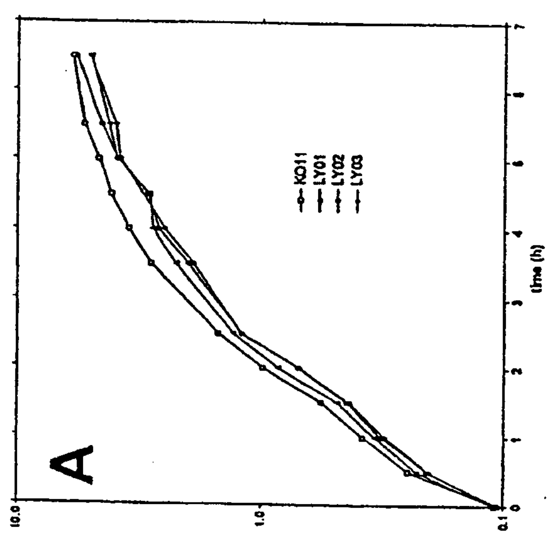


FIGURE 2A

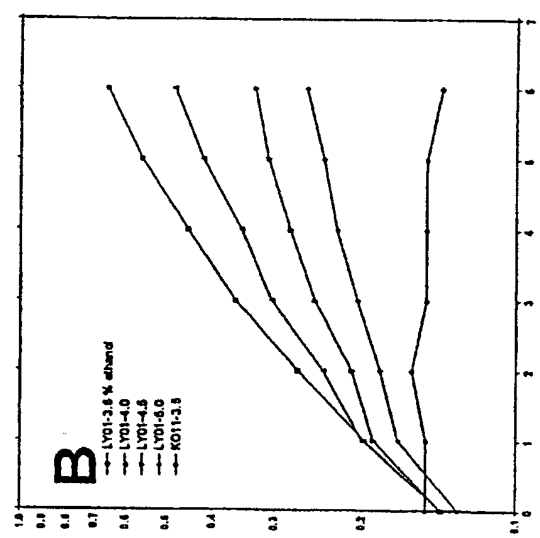


FIGURE 2B

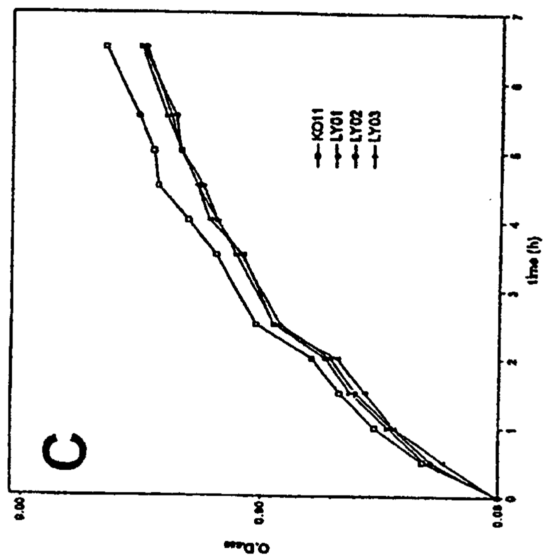


FIGURE 2C

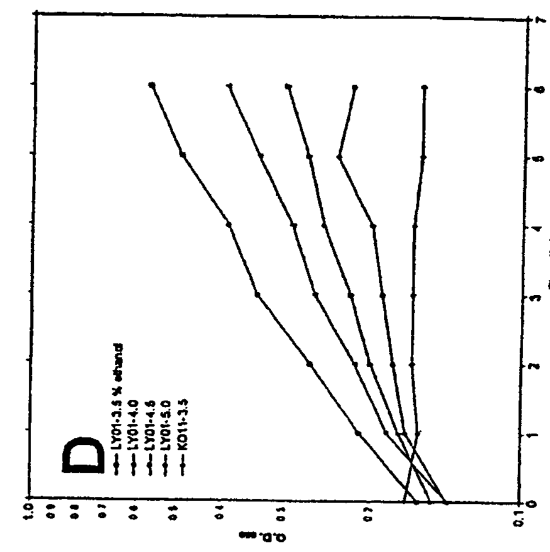


FIGURE 2D

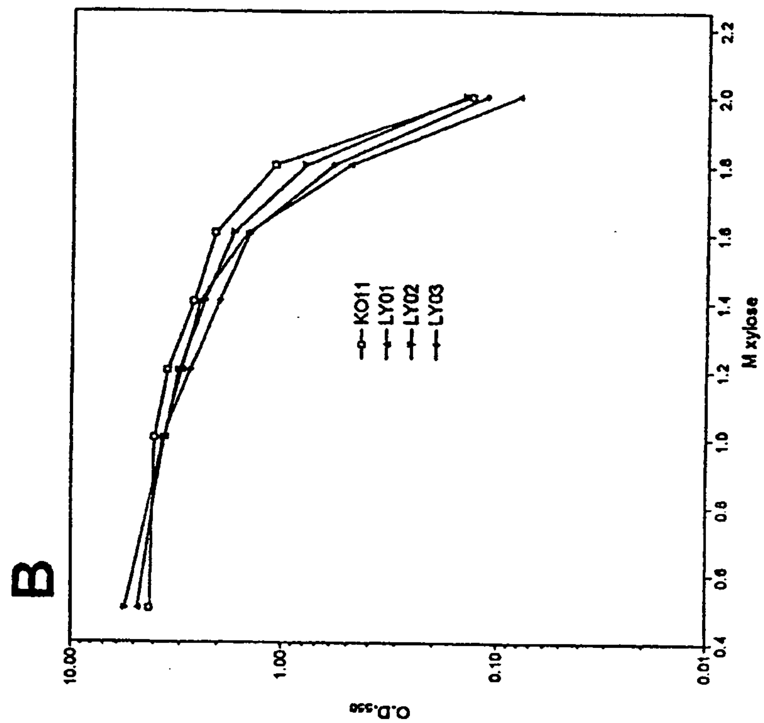


FIGURE 3B

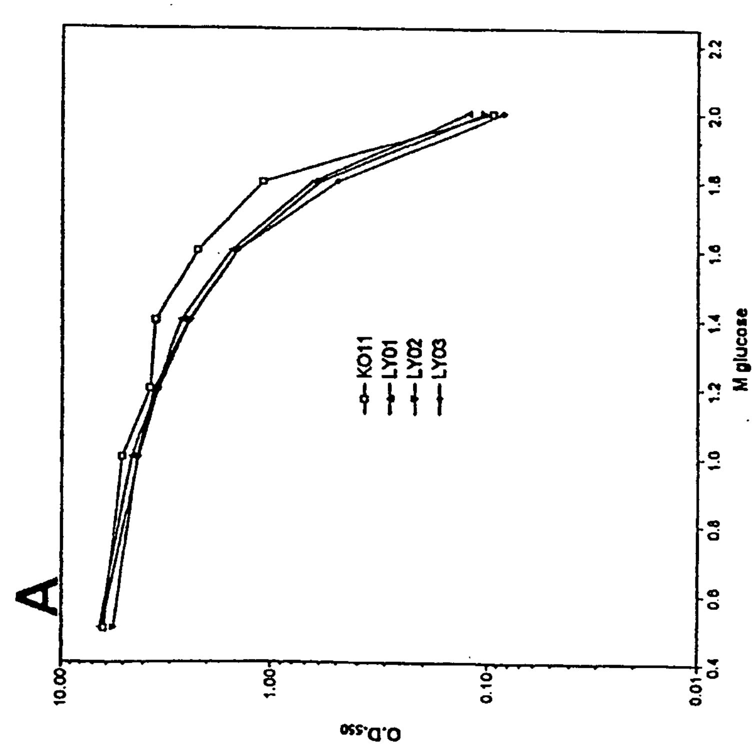


FIGURE 3A

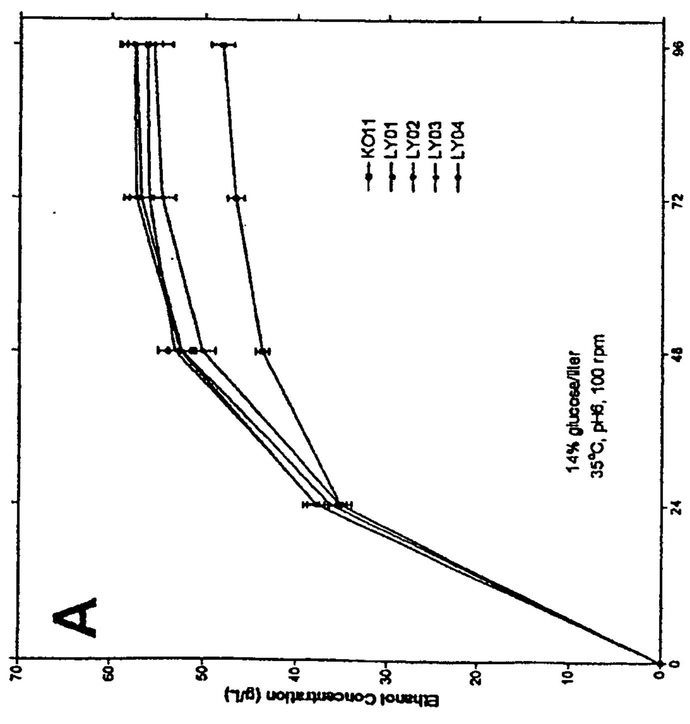


FIGURE 4A

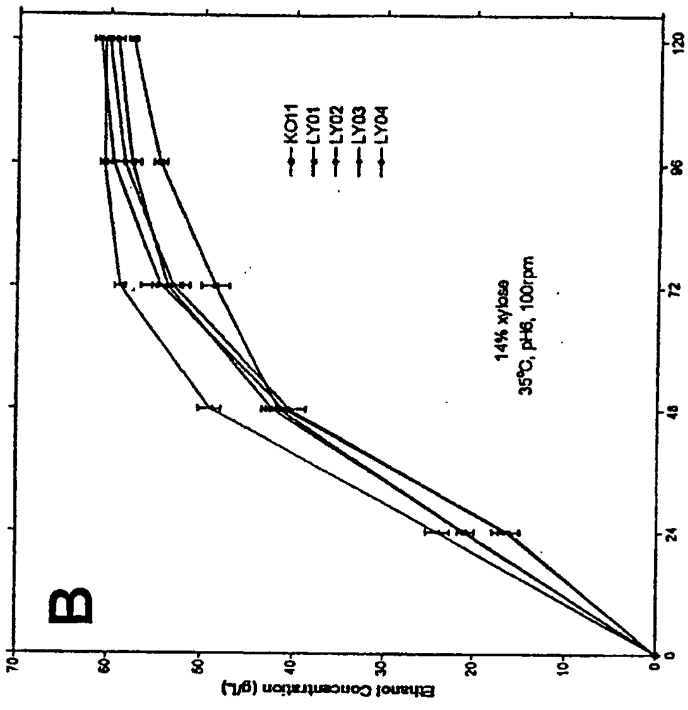
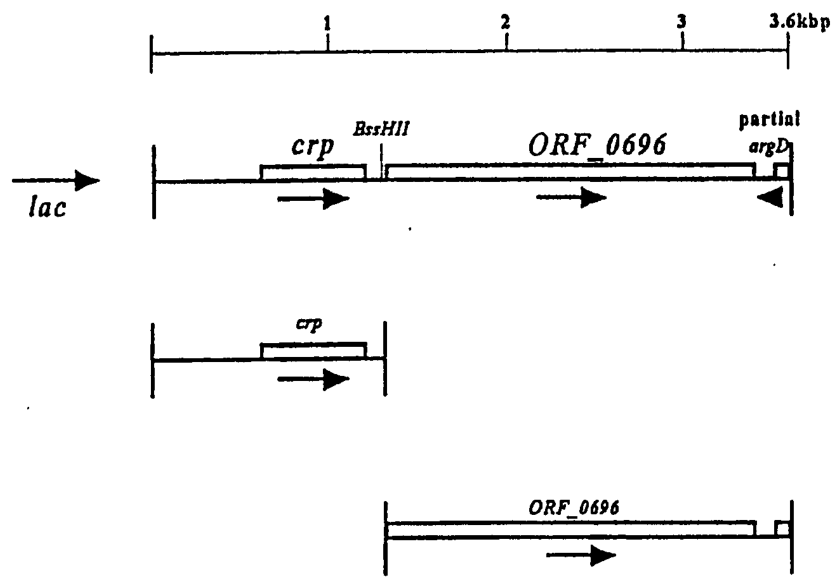


FIGURE 4B



Growth of LY03 Containing Plasmids

	% inhibition	
	LB-glucose	LB-glucose + 4% ethanol
pLOI1531	2	71
pLOI1532	0	64
pLOI1533	4	1
pUC18 control	0	0

FIGURE 5

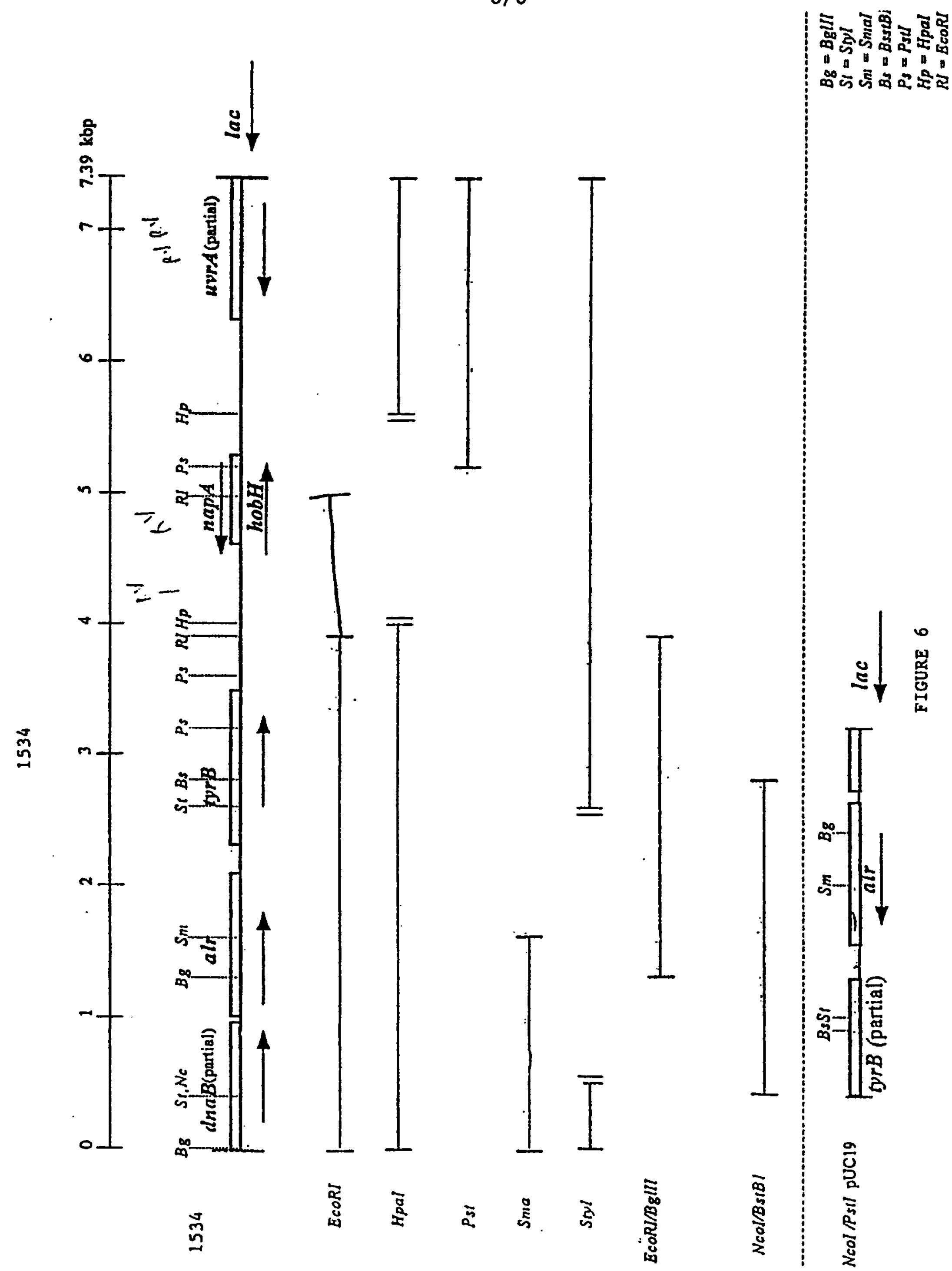

1534
0
1
2
3
4
5
6
7
7.39 kbp
lac
uvrA (partial)
napA
hobH
tyrB
alr
dnaB (partial)
Bg
St
Nc
Sm
Bs
Ps
RI
Hp
1534
EcoRI
HpaI
PstI
Sma
StyI
EcoRI/BglII
NcoI/BstBI
NcoI /PstI pUC19
tyrB (partial)
Bg = BglII
St = StyI
Sm = SmaI
Bs = BstBI
Ps = PstI
Hp = HpaI
RI = EcoRI

FIGURE 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/06405

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/01 C12P7/06 C12N1/36 //(C12P7/06,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	YOMANO L.P. ET AL.: "Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production." JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY, vol. 20, no. 2, February 1998, pages 132-138, XP002072365 see the whole document ---	8-29
X	FR 2 477 572 A (UNISEARCH LTD) 11 September 1981 see page 5, line 19 - page 6, line 7 ---	8,9,15, 16,28

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
22 July 1998	03/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

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